AD_____

Award Number:
W81XWH-07-1-0501

TITLE:
Enhancing Hormonal Therapy for Breast Cancer by Combination with a Well-Known Approved Pharmaceutical with Little Toxicity

PRINCIPAL INVESTIGATOR:
Peter Kushner

CONTRACTING ORGANIZATION:
The UNIVERSITY OF CALIFORNIA
SAN FRANCISCO, CA 94143-0962

REPORT DATE:
July 2008

TYPE OF REPORT:
Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-07-2008	Annual	01 July 2007 – 30 June 2008

4. TITLE AND SUBTITLE
Enhancing Hormonal Therapy for Breast Cancer by Combination with a Well-Known Approved Pharmaceutical with Little Toxicity

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-07-1-0501

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Peter Kushner

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The UNIVERSITY OF CALIFORNIA

SAN FRANCISCO, CA 94143-0962

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The beneficial effects of hormonal therapy with antiestrogens or aromatase inhibitors for estrogen receptor (ER) positive metastatic breast cancer are limited by the phenomenon of hormonal resistance. We have found that valproic acid (VPA), a pharmaceutical long used as an anti-convulsant and recently found to have anti-tumor action possibly because it is a histone deacetylase inhibitor, enhances the action of antiestrogens and aromatase inhibitors on breast cancer cells. Our specific hypothesis is that VPA, will enhance the effectiveness and duration of hormonal therapy of human breast cancer xenografts growing in mice and with few side effects. Thus VPA would be an immediate candidate as an agent to enhance the effectiveness of hormonal therapy in humans. In the first few months of this award we have begun to investigate the ability of VPA to enhance the actions of tamoxifen or letrozole on human breast cancer xenografts growing on nude mice. We have also begun planning a human clinical trial of the ability of VPA to enhance tamoxifen action during a two week treatment after biopsy and prior to surgery.

15. SUBJECT TERMS
Estrogen receptor positive, breast cancer, tamoxifen, aromatase, letrozole, histone deacetylase, mice xenografts, MCF-7

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	21	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Introduction:

First year annual report: Enhancing Hormonal Therapy for Breast Cancer by Combination with Valproic Acid a Well-Known Approved Pharmaceutical with Little Toxicity. Peter Kushner, PI

The beneficial effects of hormonal therapy with antiestrogens or aromatase inhibitors for estrogen receptor (ER) positive metastatic breast cancer are limited by the phenomenon of hormonal resistance. We have found that valproic acid (VPA), a pharmaceutical long used as an anti-convulsant and recently found to have anti-tumor action possibly because it is a histone deacetylase inhibitor, enhances the action of antiestrogens and aromatase inhibitors on breast cancer cells. Furthermore valproic acid prevents the pro-proliferative effects of tamoxifen on uterine cells, suggesting that the combination of tamoxifen and valproic acid might be both more effective and less potentially harmful than tamoxifen mono-therapy. VPA is currently dosed to reach a target concentration in patient serum. VPA treatment at the recommended concentrations enhances the actions of tamoxifen, raloxifene, or fulvestrant to prevent proliferation of estrogen receptor positive breast cancer cells in culture. Thus tamoxifen blocks cell cycling in these cells and VPA both potentiates tamoxifen and makes it more efficient by cooperating with it to induce apoptosis. Our specific hypothesis is that VPA, will enhance the effectiveness and duration of hormonal therapy of human breast cancer xenografts growing in mice and with few side effects. Thus VPA would be an immediate candidate as an agent to enhance the effectiveness of hormonal therapy in humans.

Body:

Statement of Work (SOW) for Enhancing Hormonal Therapy for Breast Cancer by Combination with a Well-Known Approved Pharmaceutical with Little Toxicity. Peter Kushner, PI

Task 1. Set up a micro assay to measure the concentration of valproic acid in mouse serum. And, Task 2. With the micro-assay in hand evaluate the best way to deliver valproic acid to mice.

STATUS:While we were waiting for the money to be available another group at John's Hopkins explored the means to deliver valproic acid to mice and published a paper demonstrating that the best effects on prostate cancer were achieved by delivery in the drinking water. Delivery by injection or oral gavage was very inefficient because of rapid clearence. We have been using VPA in the drinking water for our mouse experiments with breast cancer.

Our recent cell culture studies have revealed that VPA need not be present continually to induce apoptosis of breast cancer cells. Between 8 and 16 hours of exposure in a 24 period is fully effective. When valproic acid is delivered to mice in drinking water it is rapidly cleared when the mice cease drinking. Thus they are only exposed for their waking hours. This delivery system mimics the schedule that we have seen to be effective in cell culture.

If VPA is effective in mice when delivered in drinking water and only present for part of the 24 interval we will exploit a similar schedule of dosing in human studies that we contemplate will begin late this year. We have been planning a human clinical trial of tamoxifen plus valproic

acid versus tamoxifen alone with Dr. Shelly Hwang here in the Department of Surgery at UCSF. This will be a two week window trial of treatment with the drugs between biopsy and surgery. VPA can cause sleepiness and this is a complaint of some patients who have taken the drug in clinical trials for cancer. To minimize this problem we are contemplating a once a day dosing prior to bedtime. This would produce a window of drug exposure primarily during sleep. The mouse studies which model exposure for only part of a day will be relevant to the decision whether to adopt this dosing strategy.

Task 3. With a reliable method for delivery of valproic acid to mice that achieves serum concentrations in the range of 300-750 micro-molar evaluate the effect of valproic acid on the initial tumor regression of MCF-7 xenografts subjected to tamoxifen or estrogen withdrawal treatment.

Task 3a. Establish MCF-7 xenografts in ovexed nude mice purchased from Charles River. There will be a 2-4 month lag time to receive the mice from the supplier. Getting the full complement of xenografts started and up to 400 cubic millimeters will take around 3 months after the mice are in hand.

Task 3b. Remove the supplemental estrogen and exposure the mice to vehicle, tamoxifen, VAP, or tamoxifen plus VAP and measure tumor volume for the next 30 days.

Task 3 c. Remove the tumors and analyze them for growth, cell proliferation, apoptosis and gene expression of candidate genes involved in cell cycling and apoptosis. Also measure VPA levels in the tumor and assay for histone acetylation.

Task 3 d. Analyze the results and write them up. Task 3 will proceed from months 6 to 18 of the project.

STATUS: We have made one slight alteration to the protocol, which is to use MCF-7 clone 18 cells that overexpress HER2/neu for this experiment. These cells (unlike parental MCF-7 cells) are stimulated to grow by tamoxifen and will give a clearer picture whether VPA enhances the effects of tamoxifen on human breast cancer. Task 3a has been accomplished and task 3b is underway.

Task 4. Measure the time to tumor progression of MCF-7 xenografts growing on ovexed nude mice and treated with either tamoxifen or estrogen withdrawal in combination VPA as compared to no VPA treatment.

Task 4a. Establish tumors, start treatment and continue to measure tumors until progression is observed or one year passes. There will be five groups of mice 12 mice per group and two tumors per mouse. One group will get continued estrogen treatment the other four will be treated with nothing, tamoxifen, VPA, or the combination of tamoxifen and VPA. The tumors will be measured until they have at least grown to 1cm or the experiment is terminated.

Task 4b. Analyze the results of the experiment and write up. If studies on gene expression, VPA content, proliferation or apoptosis are indicated do these assays and add to the analysis. Task 4 will go from the begging of the second year to the end of the project.

STATUS: task 4 has not yet begun

Task 5. Examine the initial response of tumors to letrozole or to letrozole plus VPA. This task is similar to task 3 above and will go on in parallel to that task. We have experience in the xenograft model using standard MCF-7 cells and estrogen supplementation to initiate tumor growth. We will have to explore experimental conditions to establish the model using MCF-7 aromatase cells which can be supplemented with adrenal androgens rather than with estrogen. We are familiar with this model in cell culture but not in xenografts. Thus there may be a slight delay in this task compared to task 3. Like task 3 this task will issue in a publication, but it is likely to be one separate from that coming from task 3.

STATUS: We have extended our studies in cell culture of aromatase inhibitors to anastrozole and exemestane. They give similar results to letrozole. We have initiated Task 5 by inoculating MCF-7aro cells (engineered to express aromatase) into 5 test mice. Since we are not experienced with this model, we wished to test the tumor take efficiency before setting off on a larger experiment.

Key Research Accomplishments:

* Confirmed the generality of the observation that valproic acid enhances hormonal therapies with aromatase inhibitors.

* Decided on a reasonable way to deliver valproic acid to mice

* Begun planning and completed a draft of a protocol for a human clinical study of the ability of valproic acid to induce apoptosis of human breast cancer in a "window" clinical trial.

Reportable Outcomes:

Publication-

Breast Cancer Res Treat. 2007 Nov;105(3):297-309. Epub 2006 Dec 21.Inhibition of histone deacetylase enhances the anti-proliferative action of antiestrogens on breast cancer cells and blocks tamoxifen-induced proliferation of uterine cells.Hodges-Gallagher L, Valentine CD, Bader SE, Kushner PJ.
Department of Medicine, University of California, P. O. Box 1640, San Francisco,
CA 94143, USA.

Abstract and Podium Presentation - ERA OF HOPE 2008 Baltimore Maryland

Abstract is appended.

Conclusion:

It is too early to draw a general conclusion from this work other than that valproic acid appears promising as a agent to enhance hormonal therapy for estrogen receptor positive breast cancer. At the end of the study a more general conclusion may be possible.

References:

1: Hodges-Gallagher L, Valentine CD, Bader SE, Kushner PJ.
Inhibition of histone deacetylase enhances the anti-proliferative action of antiestrogens on breast cancer cells and blocks tamoxifen-induced proliferation of uterine cells.
Breast Cancer Res Treat. 2007 Nov;105(3):297-309. Epub 2006 Dec 21.

Abstract and Podium Presentation

Enhancing Hormonal Therapy for Breast Cancer by Combination with Valproic Acid a Well-Known Approved Pharmaceutical with Little Toxicity. Peter Kushner, PI

The beneficial effects of hormonal therapy for estrogen receptor (ER) positive metastatic breast cancer are limited by the phenomenon of hormonal resistance. Similarly hormonal therapy to prevent recurrence after successful surgery is not always effective. One attractive tactic to extend the magnitude and duration of response to hormonal therapy would be to combine it with another therapy with an independent target in the tumor cells. Unfortunately, the most familiar of such candidate therapies, cytotoxic agents used in chemotherapy, do not combine effectively with hormonal therapy which appears to antagonize the beneficial actions of chemotherapy as observed both in clinical trials and in cell culture models.

We have found that valproic acid (VPA), a pharmaceutical long used as an anti-convulsant and recently found to have anti-tumor action possibly because it is a histone deacetylase inhibitor, enhances the action of antiestrogens and aromatase inhibitors. Furthermore valproic acid prevents the pro-proliferative effects of tamoxifen on uterine cells, suggesting that the combination of tamoxifen and valproic acid might be both more effective and less potentially harmful than tamoxifen mono-therapy. VPA is currently dosed to reach a target concentration in patient serum. VPA treatment at the recommended concentrations enhances the actions of tamoxifen, raloxifene, or fulvestrant to prevent proliferation of estrogen receptor positive breast cancer cells in culture. Thus tamoxifen blocks cell cycling in these cells and VPA both potentiates tamoxifen and makes it more efficient by cooperating with it to induce apoptosis. Our specific hypothesis is that VPA, will enhance the effectiveness and duration of hormonal therapy of human breast cancer xenografts growing in mice and with few side effects. Thus VPA would be an immediate candidate as an agent to enhance the effectiveness of hormonal therapy in humans.

Specific Aims: Aim 1. To examine the ability of VPA to enhance both **a)** the initial response and **b)** the duration of response to the antiestrogen tamoxifen or to estrogen ablation therapy of MCF-7 xenografts grown in ovariectomized nude mice, and to study the effects of VPA on markers of cell cycling, apoptosis, and gene expression in the xenografts. **Aim 2.** To examine the ability of VPA to enhance the extent of initial response and duration of response to the aromatase inhibitor letrozole of MCF-7/aromatase cells growing as xenografts under stimulation with adrenal androgen in ovex nude mice.

We will present studies showing both the effect and potential mechanisms whereby VPA enhances hormonal therapy in cell culture and (if ready) some preliminary results of our studies in mice.

Breast Cancer Res Treat
DOI 10.1007/s10549-006-9459-6

PRECLINICAL STUDY

Inhibition of histone deacetylase enhances the anti-proliferative action of antiestrogens on breast cancer cells and blocks tamoxifen-induced proliferation of uterine cells

Leslie Hodges-Gallagher · Cathleen D. Valentine · Suzy El Bader · Peter J. Kushner

Received: 7 November 2006 / Accepted: 13 November 2006

Abstract Here we report a novel potential therapeutic strategy using histone deacetylase (HDAC) inhibitors to enhance the action of hormonal therapy agents in estrogen receptor alpha (ERα)-positive breast cancer. HDAC inhibitors [trichostatin A (TSA), suberoylanilide hydroxamic acid (SAHA) and valproic acid (VPA)], inhibited proliferation of MCF-7 breast cancer cells and, in combination with tamoxifen inhibited proliferation better than with either agent alone. VPA, an anti-convulsant drug with HDAC inhibitory activity, enhanced tamoxifen action at doses within the concentration range used for anti-convulsive therapy. VPA cooperated with tamoxifen in a variety of ERα-positive cell lines and was also effective when combined with other antiestrogens, and with aromatase inhibition. VPA enhanced antiestrogen action by promoting cell death via apoptosis without affecting cell cycling. Some of this action may be due to VPA's ability to induce the pro-apoptotic gene Bik, which is also induced by antiestrogens. Remarkably, VPA blocked the undesirable pro-proliferative action of tamoxifen on uterine endometrial cells. Our in vitro results suggest that VPA and other HDAC inhibitors have the potential to enhance hormonal therapy for ERα-positive breast cancer and simultaneously reverse the adverse effects of antiestrogens in the uterus.

Keywords Histone deacetylase inhibitors · Tamoxifen · Antiestrogen · Breast · Uterus

L. Hodges-Gallagher · C. D. Valentine · S. E. Bader · P. J. Kushner (✉)
Department of Medicine, University of California, P. O. Box 1640, San Francisco, CA 94143, USA
e-mail: kushner@itsa.ucsf.edu

Introduction

Significant improvements in endocrine therapy for breast cancer have occurred with the introduction of aromatase inhibitors and fulvestrant, an agent that down-regulates estrogen receptor (ER) expression. Unfortunately, like tamoxifen, a subset of tumors are either initially resistant or acquire resistance over time, despite maintaining ERα expression [1–3]. In addition to resistance, tamoxifen therapy has undesirable side effects such as an increase in venous thromboembolic events and stimulating proliferation of uterine endometrial cells, putting women at a higher risk for developing uterine adenocarcinoma [4]. Thus, there is a compelling need to develop new therapeutic strategies to circumvent resistance against hormonal therapy in breast tumors and extend the disease-free survival of patients.

One attractive therapeutic strategy to improve the efficacy of hormonal therapies is to combine them with agents that target separate biochemical pathways. Although several randomized trials have combined antiestrogens concomitantly with traditional adjuvant chemotherapies that target the integrity of DNA, these trials have produced disappointing results, perhaps because hormonal therapy antagonizes the effectiveness of chemotherapy [5, 6]. Such antagonist action of hormonal therapy is seen in cell culture, and may result both from antiestrogen effects on cell cycling and independent actions of the antiestrogen [7–9]. A recent popular strategy has been to combine hormonal therapy with agents that block signaling from growth factor receptors, including the epithelial growth factor receptor (EGFR), the insulin-like growth factor receptor (IGFR), and their down stream effectors such

as extracellular signal-regulated kinase (ERK), AKT/protein kinase B, and the mammalian target of rapamycin (mTOR). Combination of hormonal therapy and inhibitors of these pathways has shown promise in cell culture and preclinical animal studies [10]. However, a recent trial of combining the aromatase inhibitor letrozole with temsirolimus, an inhibitor of mTOR, the downstream effector of IGF signaling, was terminated for lack of benefit [11].

An alternative unexplored approach to maximize the efficacy of antiestrogens and aromatase inhibitors in ERα-positive tumors is to combine these therapies with agents that target events in gene expression that parallel the actions of antiestrogens. Histone deacetylases (HDAC) represent a key component of transcriptional control of ER signaling and increase the transcriptional activity of tamoxifen [12–14]. There are a number of small molecule inhibitors of HDACs in preclinical development that have been shown to inhibit proliferation, induce differentiation and/or apoptosis in a wide range of cancer cells and hold much promise as anti-neoplastic agents [15, 16]. Hyperacetylation is associated with a transcriptionally permissive environment and HDAC inhibitors, which ends up only affecting a small number of target genes, activate genes involved in cell cycle arrest, apoptosis, and differentiation [17–19]. Furthermore, HDAC inhibitors increase the efficiency of several anticancer drugs that target the DNA [20, 21]. Several studies have reported that HDAC inhibitors inhibit proliferation of ERα-positive breast cancer cells in vitro as well as tumor growth in a rat mammary carcinoma model [19, 22, 23]. The mechanism is unclear but may in part involve down-regulation of ERα, the ER that drives proliferation in breast cancer cells [24, 25].

The anti-convulsant and mood stabilizer valproic acid (VPA) was recently discovered to inhibit classes I and II HDAC activity at therapeutic concentrations [26, 27]. In contrast to other HDAC inhibitors, VPA is well tolerated and has been safely used in patients for long-term treatment of epilepsy and bipolar disorders for more than 35 years. VPA inhibits the proliferation of many types of cancer cells including breast cancer cells [24, 28, 29]. In vivo, VPA inhibited tumor growth and delayed lung metastasis in the MT-450 rat breast cancer model [27]. VPA also synergizes with traditional chemotherapy agents to inhibit the proliferation of tumor cells in culture and in xenografts [30]. Because of these promising studies, several clinical studies have commenced to investigate the effective dose of VPA to achieve an anti-neoplastic effect in solid tumors, including breast cancer and also in combination with traditional chemotherapy agents [31].

Here we report that the combination of VPA and hormonal therapy inhibits the proliferation of ERα-positive breast cancer cells better than using either agent alone. Surprisingly, VPA also dramatically inhibits the proliferative effects of tamoxifen on uterine endometrial cells. Our results suggest that VPA may confer protection against the undesirable pro-proliferative effects of tamoxifen on the uterus while increasing the anti-proliferative effects of antiestrogens and aromatase inhibitors against E2-sensitive breast tumors.

Materials and methods

Cell culture and ligands

MCF-7 cells were provided by C. Walker (MD Anderson Cancer Center, Houston, TX, USA) and were routinely cultured in IMEM (Invitrogen, Grand Island, NY, USA) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA). Ishikawa cells were also provided by C. Walker and cultured in DMEM/Ham's F12 with 10% FBS. T47-D cells were obtained from F. Schaufele (University of California, San Francisco, CA, USA) and were routinely cultured in DMEM (Invitrogen) plus 10% FBS. ZR-75-1 cells were provided by B. Hahn (University of California, San Francisco) and were routinely cultured in RPMI (Invitrogen) plus 10% FBS. MCF-7/neo and MCF-7/HER2 (clone 18) cells were also obtained from B. Hahn and cultured in DMEM plus 10% FBS. MCF-7aro cells were provided by S. Chen (Beckham Research Institute of the City of Hope) and routinely grown in DMEM/Ham's F12 plus 10% FBS. For all experiments cells were switched to phenol red-free media containing 5% charcoal/dextran-stripped FBS (Hyclone) for 3–5 days prior to start of the experiment. Cells were treated with ligands in media containing 2–5% stripped FBS for the indicated times. The 17β-estradiol (E2), 4-trans-hydroxytamoxifen (OH-Tam), raloxifene, trichostatin A (TSA), and VPA sodium salt (sodium 2-propylpentanoate) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fulvestrant (ICI 182,780) was obtained from Tocris (Ellisville, MO, USA) and suberoylanilide hydroxamic acid (SAHA) from BioVision (Mountain View, CA, USA).

Cell proliferation assays

Cells growing in 24-well dishes were treated with ligands in triplicate for the indicated times. Cells were trypsinized and counted electronically with a Coulter

Counter (Coulter Electronics, Hialeah, FL, USA). Alternatively, cell proliferation was measured using a fluorescent DNA-binding assay, CyQUANT (Invitrogen) in which cells were treated with ligands in triplicate in 96-well plates for the indicated times and assayed according to the manufacturer's instructions.

Flow cytometry

MCF-7 cells growing in 100 mm dishes were treated with ligands for 48 h and then trypsinized. Both floating and attached cells were centrifuged and fixed in 70% ethanol and stained with 50 mg/ml propidium iodide (Roche, Nutley, NJ, USA). DNA content was obtained by measuring 10,000 events on a FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The percentages of cells in each phase of the cell cycle were determined using the Watson (Pragmatic) model analyzed by FlowJo flow cytometry software (Treestar, San Carlos, CA, USA).

Apoptosis

MCF-7 cells growing on coverslips were treated with ligands for 72 h and unfixed cells were assayed with the AnnexinV-FLOUS Staining kit (Roche), according to manufacturer's instructions. Total cell number was determined by counting nuclei stained with Hoescht 33342 (Roche). Fluorescence was analyzed using a Zeiss Axioplan fluorescent microscope (Zeiss, Thornwood, NY, USA).

Transfections

MCF-7 cells growing in 12-well dishes were transfected with ERE-Luc and β-galactosidase reporter gene using Lipofectamine 2000 transfection reagent (Invitrogen) according to manufacturer's instructions. ERE-Luc reporter gene has been previously described [32, 33]. Five hours after transfection, cells were treated in triplicate with ligands and harvested 24 h later using a lysis buffer containing 100 mM Tris–HCl, 1% Triton-X100 and 1 μl/ml dithiothreitol. Reporter gene activity was measured using assay kits for Luciferase (Promega, Madison, WI, USA) and β-galactosidase (Tropix, Bedford, MA, USA), according to manufacturer's instructions.

Immunoblotting

Cells were grown in 100 mm dishes and treated with ligands in triplicate. Cells were harvested 24 h later using a lysis buffer containing 65.2 mM Tris–HCl, 154 mM NaCl, 1:100 NP-40, 1:400 sodium doxycholate, 2 mM sodium orthovanadate, 1 mM sodium flouride, 1 mM phenylmethylsulphonylfluoride, and 1 μg/ml each of leupeptin, aprotinin, and pepstatin. Whole cell extracts were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted using standard methods with the following antibodies: ERα, Bik, Bcl-2, actin and β-tubulin (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), cyclin D1 and acetylated histone H4 (Zymed, San Francisco, CA, USA).

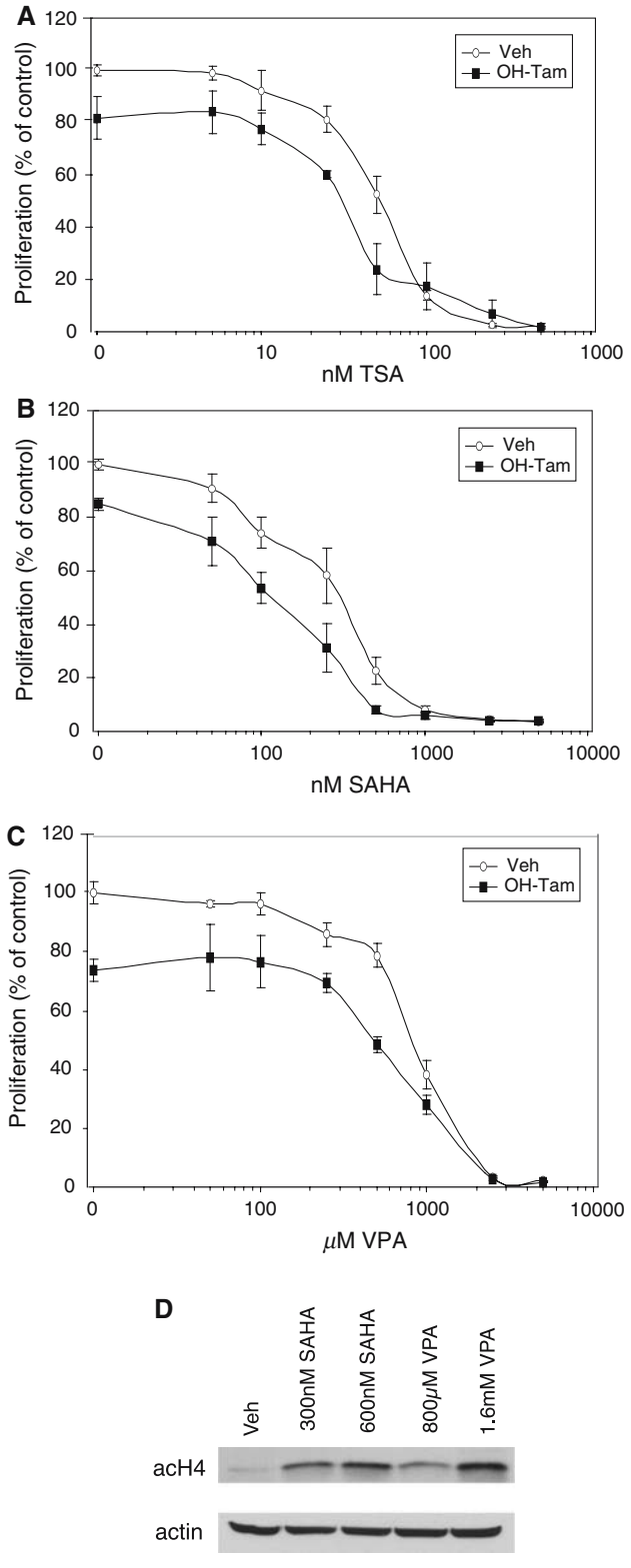
Statistical analysis

All results are presented as mean ± standard error (SEM). Statistical significance between treatment groups was determined by ANOVA with Fisher's planned least significant test at $P \leq 0.05$ conducted using Statview software (SAS Institute, Cary, NC, USA).

Results

HDAC inhibitors enhance tamoxifen action on breast cancer cells

Since HDAC inhibitors have been previously shown to inhibit proliferation of ERα-positive breast cancer cells, we examined their ability to cooperate with antiestrogens, i.e., inhibiting cell proliferation better when used in combination than using either agent alone. The well-described HDAC inhibitor, TSA, inhibited E2-induced proliferation of MCF-7 breast cancer cells in a dose responsive manner and co-treatment with the biologically active metabolite of tamoxifen, 4-hydroxytamoxifen (OH-Tam), inhibited proliferation better than either agent alone (Fig. 1a). The IC_{50} of TSA alone was 51 nM and co-treatment with tamoxifen shifted the IC_{50} to 32 nM—thus, tamoxifen enhanced the potency as well as efficacy of TSA. Similarly, the HDAC inhibitor SAHA inhibited MCF-7 cell growth, and tamoxifen co-treatment shifted the IC_{50} of SAHA from 300 to 125 nM (Fig. 1b). We also examined the effects of VPA, an anti-convulsant recently shown to possess HDAC inhibitory activity [26, 27]. Like the other HDAC inhibitors, VPA inhibited MCF-7 cell growth and tamoxifen co-treatment shifted the IC_{50} of VPA from 800 to 500 μM. Thus, all three HDAC inhibitors enhanced the efficacy of tamoxifen, and tamoxifen enhanced the potency of the HDAC inhibitors, suggesting that HDAC inhibitors in general cooperate with the anti-proliferative effects of tamoxifen.



◀ **Fig. 1** HDAC inhibitors inhibit MCF-7 cell proliferation and cooperate with the anti-proliferative effects of tamoxifen. MCF-7 cells were stimulated with 100 pM 17β-estradiol (*E2*) and treated with a range of doses of TSA (**A**), SAHA (**B**) or VPA (**C**), alone and in the presence of 10 nM 4-hydroxytamoxifen (*OH-Tam*). Proliferation was measured on day 7 using a fluorescent DNA-binding assay and values represent the percentage of fluorescence of 100 pM E2 alone (control) and *error bars* represent the SEM from triplicate wells. (**D**) Increased acetylation of histone H4 by HDAC inhibitors at doses that inhibit breast cancer proliferation. MCF-7 cells were treated for 48 h with SAHA and VPA at the indicated doses and immunoblotted with an antibody specific for the acetylated form of histone H4

Next we determined if HDAC inhibitors increase the acetylation of histones at doses observed to inhibit breast cancer cell proliferation by immunoblotting with an antibody specific for the acetylated form of histone H4. MCF-7 cells treated for 48 h with SAHA exhibited increased acetylated H4 protein with 300 nM, its IC_{50}, and twice this amount, 600 nM, further increased acetylated H4 levels (Fig. 1d). The IC_{50} of VPA, 800 μM, also increased acetylated H4 levels, as did 1.6 mM of VPA. It should be noted that tamoxifen treatment did not alter acetylated H4 levels by itself, nor did it alter acetylation induced by HDAC inhibitors (data not shown). These data indicate that accumulation of acetylated histones occur at doses which correspond with the anti-proliferative effect of tamoxifen, suggesting that the ability of these agents to inhibit HDAC activity contributes at least in part for the observed effects.

VPA enhances the anti-proliferative effect of tamoxifen in ERα-positive cell lines

To further characterize the interaction of antiestrogens and HDAC inhibitors on hormone sensitive cells, we focused on VPA as a model HDAC inhibitor since the pharmacology and bioavailability of VPA has been well characterized. Plasma levels of VPA in patients treated for epilepsy and bipolar disorders range from 50 to 120 μg/ml (350–850 μM), therefore we used the therapeutic dose of 750 μM of VPA to determine its effect alone and in combination with tamoxifen in E2-sensitive cells. Both VPA and tamoxifen inhibited MCF-7 cell proliferation, particularly in the presence of E2 (Fig. 2a). When VPA and tamoxifen treatment were combined, MCF-7 cell proliferation was inhibited to a greater extent than with either ligand alone. T47D cells responded similarly to MCF-7 cells, with VPA and tamoxifen cooperating in their anti-proliferative effects (Fig. 2b). ZR-75-1 cells, which exhibited a higher level of basal proliferation compared to the other two cell lines, were also significantly inhibited by co-treatment of VPA and tamoxifen in the absence of E2 (Fig. 2c). Together, these results indicate that VPA and tamoxifen cooperate in their anti-proliferative effects for ERα-positive breast cancer cells.

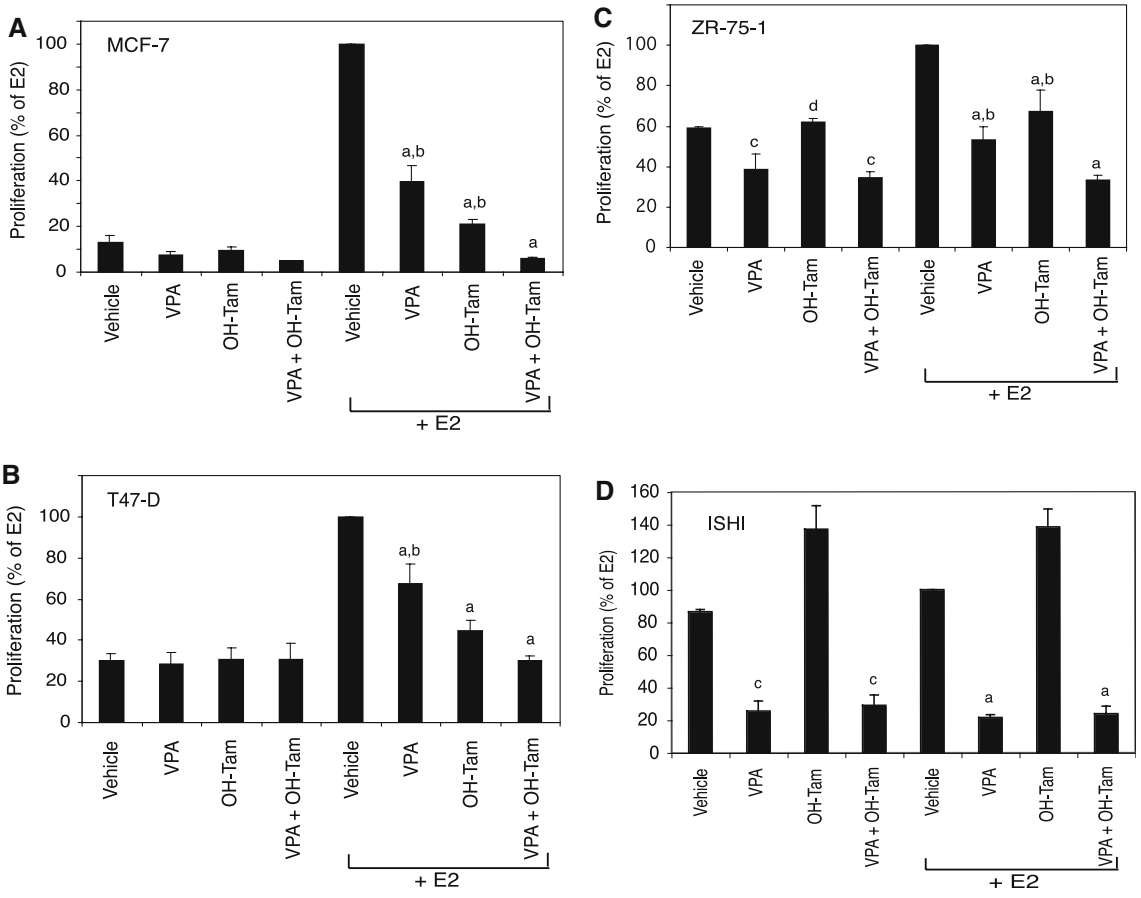


Fig. 2 VPA inhibits cell proliferation and enhances the anti-proliferative effect of tamoxifen in breast cancer cells and reverses tamoxifen-induced proliferation in uterine endometrial cells. Three ERα-positive breast cancer cell lines, MCF-7 (**A**), T47-D (**B**), and ZR-75-1 (**C**), were treated with 750 μM VPA, 10 nM OH-Tam and either 100 pM or 1 nM E2 for 6–7 days and counted electronically. *Bars* represent the average of three independent experiments presented as a percentage of E2 alone and *error bars* represent SEM from the three experiments. Statistical significance was determined by ANOVA at $P \leq 0.05$: **A** denotes statistical difference from E2 alone; **B** denotes statistical difference from E + VPA + OH-Tam; and **C** denotes statistical difference from vehicle alone; and **D** denotes statistical difference from VPA + OH-Tam. (**D**) VPA inhibits tamoxifen-induced proliferation in Ishikawa uterine endometrial cells. Ishikawa cells were treated for 6–7 days with the same concentrations of ligands as above. Cells from three independent experiments were counted and analyzed as described above

VPA reverses the agonist activity of tamoxifen in endometrial cells

One of the major drawbacks of tamoxifen therapy is the increased proliferation of uterine endometrial cells, putting patients at an increased risk of developing adenocarcinoma [4]. Any therapeutic agent that enhances the anti-proliferative effect of tamoxifen in the breast should ideally not also enhance the agonist activity of tamoxifen in the uterine endometrium. Therefore, we treated Ishikawa adenocarcinoma cells, which proliferate in response to tamoxifen, with VPA alone or combined with tamoxifen (Fig. 2d). VPA dramatically inhibited proliferation alone and equally antagonized the proliferative effect of both E2 and tamoxifen. Thus, while VPA cooperated with the anti-proliferative effect of tamoxifen in breast cells, VPA reversed the E2-like agonist activity of tamoxifen in endometrial cells.

VPA enhances the efficacy and potency of antiestrogens in breast cancer cells

Since VPA enhances the efficacy of tamoxifen we next examined whether VPA would also enhance its potency in a dose responsive proliferation assay. E2-stimulated MCF-7 cells were treated with a range of concentrations of tamoxifen, both in the presence and absence of 750 μM VPA (Fig. 3a). VPA treatment alone inhibited E2-stimulated cell proliferation by 25%

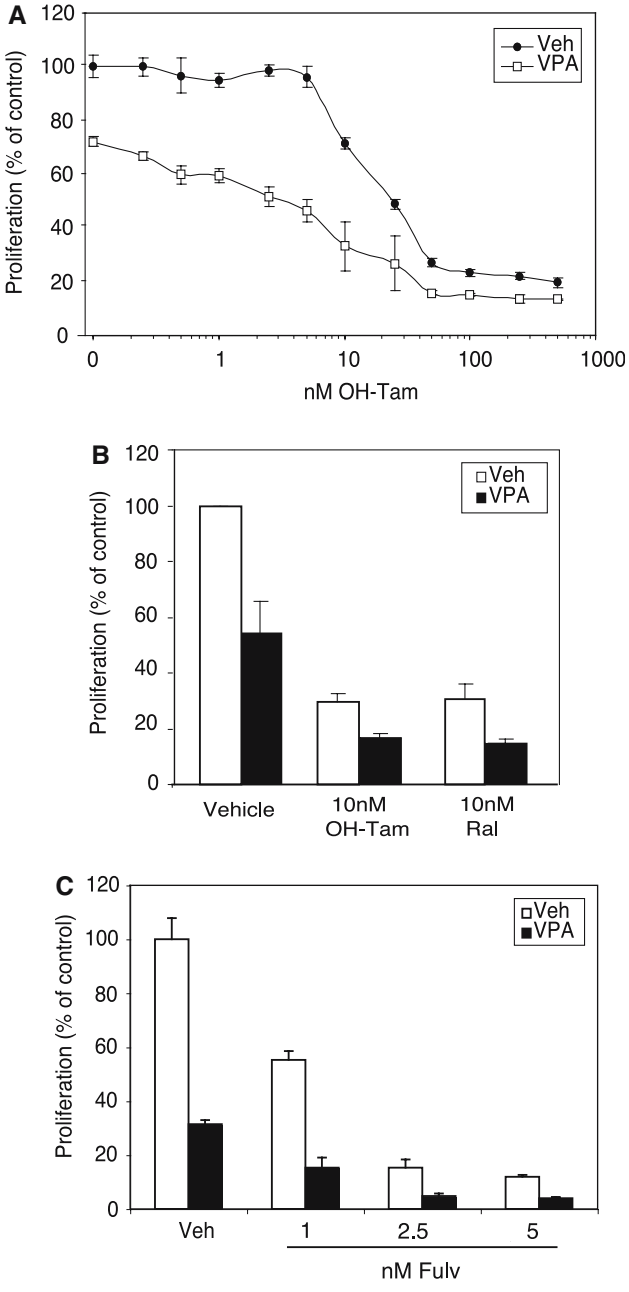


Fig. 3 VPA enhances the efficacy of tamoxifen and other antiestrogens. (**A**) VPA and tamoxifen have an additive effect in inhibiting MCF-7 cell proliferation. Cells were treated with 100 pM E2 with and without 750 μM VPA in the presence of OH-Tam, ranging from 0 to 500 nM. On day 6 of the assay, proliferation was measured using a fluorescent DNA-binding assay and values represent the percentage of fluorescence of E2 alone (control) with *bars* representing the SEM from triplicate wells in a representative experiment. (**B**) VPA enhances the efficacy of raloxifene. MCF-7 cells were treated with the indicated concentrations of OH-Tam or raloxifene (*Ral*) in the presence of 100 pM E2 and either plus or minus 750 μM VPA for 7 days and electronically counted. *Bars* represent the average proliferative response relative to E2 alone (control) from three independent experiments with *error bars* representing SEM. (**C**) VPA enhances the efficacy of fulvestrant. MCF-7 cells were treated as described in **B** with the indicated concentrations of fulvestrant (*Fulv*). Cells were electronically counted and a representative experiment is shown with *error bars* representing the SEM from triplicate wells

and increased the relative efficacy of tamoxifen at all doses tested. VPA also shifted the IC_{50} for tamoxifen treatment to 3 nM, compared to 25 nM when tamoxifen was used alone. Thus, VPA enhanced the potency as well as the efficacy of tamoxifen action on cell proliferation.

We next investigated if VPA could also be effective in enhancing the anti-proliferative activity of other antiestrogens besides tamoxifen. MCF-7 cells treated with VPA alone exhibited decreased proliferation (Fig. 3b). Similar to tamoxifen, raloxifene substantially decreased E2-stimulated growth and co-treatment with VPA further decreased cell number. The pure antiestrogen fulvestrant (also known as ICI 182,780 or faslodex) inhibited E2-stimulated growth in a dose responsive manner and VPA increased this inhibition with 1, 2.5, and 5 nM of fulvestrant (Fig. 3c). These data indicate that in addition to tamoxifen, VPA also cooperates with the anti-proliferative effects of raloxifene and fulvestrant and possibly other antiestrogens.

VPA does not arrest cell cycle progression

To determine if VPA enhanced the anti-proliferative effect of tamoxifen by halting cell cycle progression, we used flow cytometry to measure the number of cells in each phase of the cell cycle. MCF-7 cells were treated with ligands for 48 h and the population of cells in G1, S, and G2 was estimated based on DNA content (Fig. 4a). VPA treatment alone induced a small arrest in the G1 phase in the absence of E2, but had no effect when E2 was present. As expected, tamoxifen had a dramatic effect of arresting cells in G1 in the presence of E2. However, the addition of VPA to tamoxifen on E2-induced cell cycle progression did not yield a detectable change, in contrast to the cooperative inhibitory effect on cell proliferation observed above. These observations suggest that VPA may be enhancing the action of tamoxifen by some means other than altering cell cycle progression.

VPA induces apoptosis and enhances the apoptotic activity of tamoxifen

We examined MCF-7 cells with phase microscopy to determine whether VPA and tamoxifen induced morphological changes such as those associated with cells

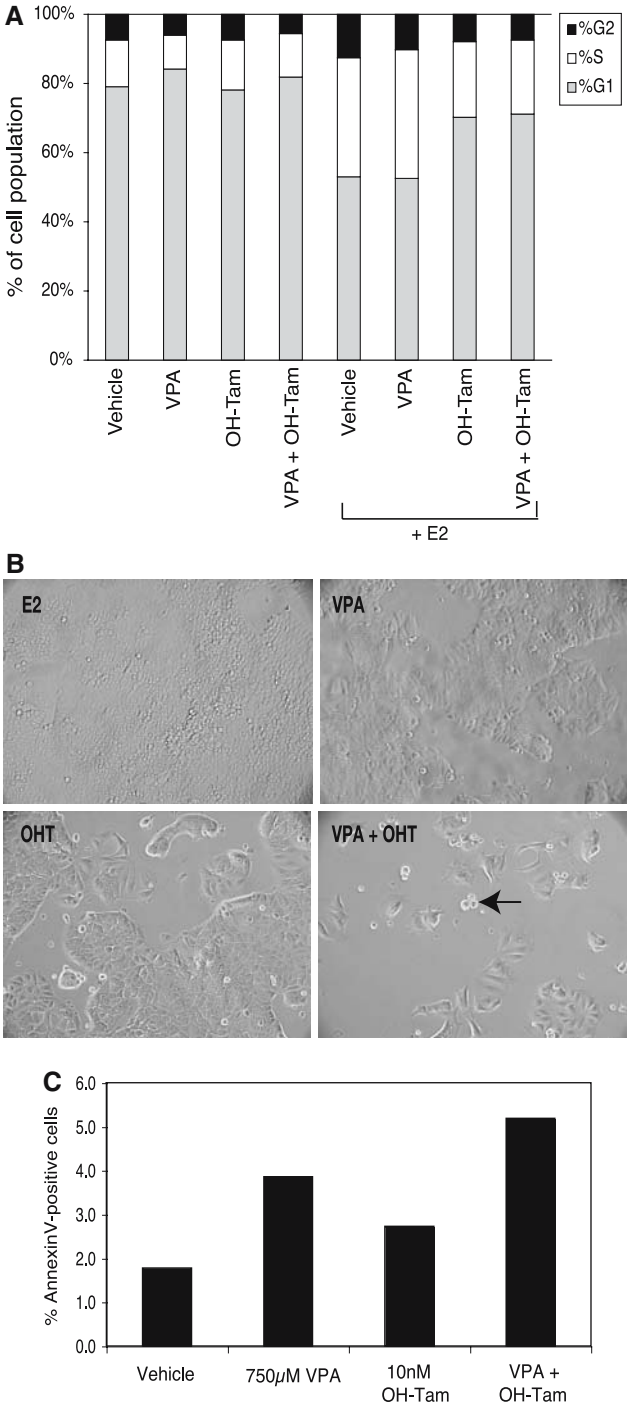


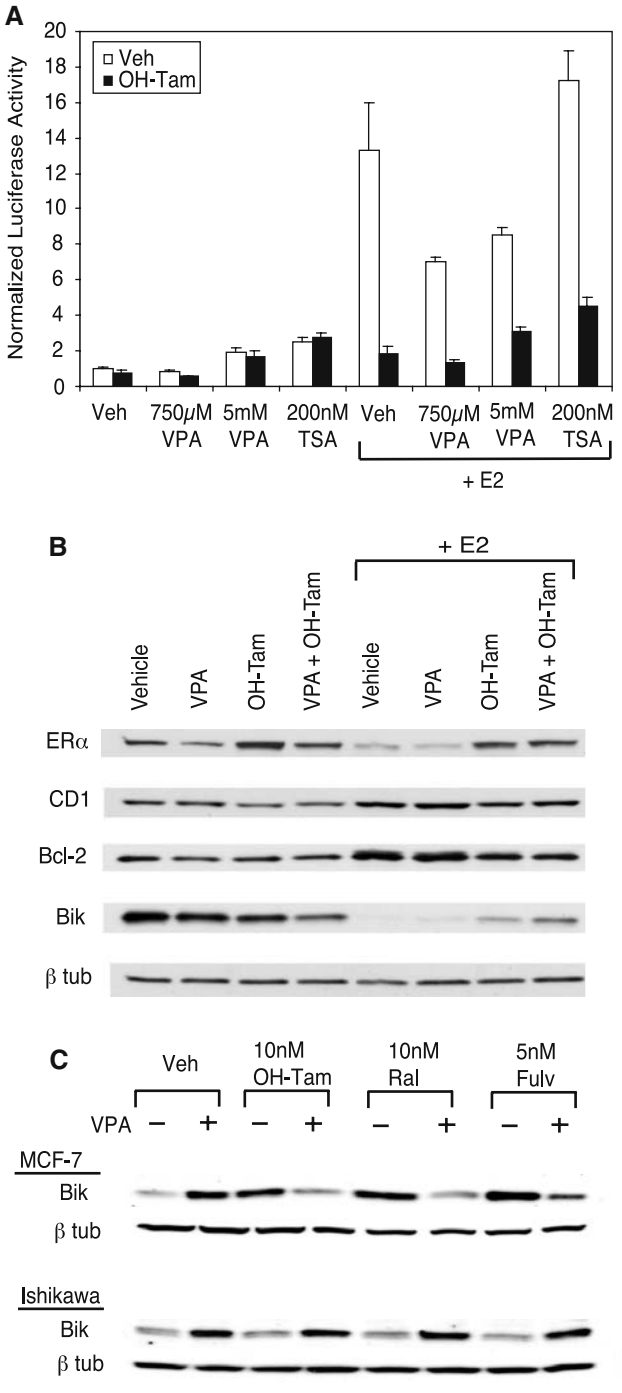
Fig. 4 Effect of VPA and tamoxifen co-treatment on cell cycling and cell death. (**A**) VPA alone or in combination with tamoxifen does not alter E2-induced cell cycle distribution. Flow cytometry was used to measure DNA content of MCF-7 cells treated for 48 h with the following concentrations of ligands as indicated: 100 pM E2, 750 μM VPA, and 10 nM OHT. (**B**) Increased cell death and apoptotic-like morphology with VPA and tamoxifen co-treatment. MCF-7 cells were treated with the same concentrations of ligands above as indicated for a 6-day period and a representative field analyzed by phase microscopy at 10× magnification. The *arrow* points to a group of cells with apoptotic-like morphology. (**C**) VPA increases apoptosis and enhances tamoxifen-induced apoptosis. MCF-7 cells were treated for 72 h in the absence of E2 and apoptotic index measured by AnnexinV-fluorescein staining. For each treatment a minimum of 5,000 nuclei, pooled from three independent experiments, were analyzed by fluorescent microscopy

undergoing apoptosis (Fig. 4b). After 6 days in culture, E2-treated cells grew into a confluent monolayer, covering virtually every available space in the culture dish. VPA-treated cultures exhibited fewer cells as well as an increased number of floating cells. A dramatic decrease in cell number was observed when VPA and tamoxifen were used together than with either drug alone. Additionally, VPA and tamoxifen co-treatment produced an increased proportion of cells exhibiting bright, condensed, and/or rounded cells with an increased number of floating cells, morphologies indicative of cells in late-stage apoptosis.

To further characterize the effect of combination treatment with VPA and tamoxifen on cell death we employed a quantitative apoptotic assay specific for annexin V, which binds to the outer membrane of unfixed cells undergoing apoptosis and is observed even at the earliest stages of apoptosis [34]. Since unstimulated MCF-7 exhibit low levels of apoptosis (1.78% of the total cell population), at least 5,000 cells were analyzed to obtain an accurate measurement of the apoptotic index (Fig. 4c). In reality, the number of annexin V-positive cells could be much higher since microscopic analysis allowed only attached cells to be analyzed, thus excluding cells undergoing late stage apoptosis that have detached in culture. Both VPA and tamoxifen treatments alone induced similar increases in the number of annexin V-positive cells observed, 3.85 and 2.72%, respectively. VPA plus tamoxifen co-treatment further increased the apoptotic index to 5.18%. In summary, VPA enhanced tamoxifen-induced apoptosis while having little or no effect on tamoxifen's ability to arrest cell proliferation.

Interactions of VPA and tamoxifen on gene expression mediated by estrogen receptor alpha (ERα)

Both antiestrogens and HDAC inhibitors are believed to exert their anti-proliferative effects in hormone sensitive cells by modulating ER activity and/or altering ER protein levels. Since ERα is a major mediator of proliferation in breast cancer cells, we analyzed the effect of VPA and tamoxifen on transcriptional activity at a luciferase reporter gene containing a consensus estrogen response element (ERE) (Fig. 5a). In



◀ **Fig. 5** Effect of VPA and tamoxifen co-treatment on ER-mediated gene expression. (**A**) VPA activates transcription and enhances tamoxifen agonist activity at an ERE reporter gene. MCF-7 cells were transiently transfected with ERE-Luc and treated with and without 10 nM OH-Tam along with the indicated concentrations of ligands for 24 h and assayed for lucifererase activity. *Bars* represent fold-induction relative to vehicle from a representative experiment and *error bars* represent the SEM from triplicate wells. (**B**) VPA and tamoxifen interaction on target genes. MCF-7 cells were treated for 72 h with vehicle or 100 pM E2, 750 μM VPA, and/or 10 nM OH-Tam and protein lysates immunoblotted with ERα, CD1, Bik, or Bcl-2 antibodies, with β-tubulin serving as a loading control. (**C**) VPA up-regulates the pro-apoptotic protein Bik. MCF-7 and Ishikawa cells were treated with 100 pM E2 and with the indicated concentrations of ligands in the presence and absence of 750 μM VPA for 6 days and protein lysates immunoblotted with Bik

transiently transfected MCF-7 cells endogenous ER stimulated transcription approximately 13-fold with 100 pM E2 treatment. High concentrations of TSA and VPA alone induced transcription and also increased the transcriptional activity of tamoxifen, as seen previously by us and others [13, 14, 35]. However, 750 μM VPA, the dose previously demonstrated to inhibit tamoxifen induced growth, was unable to activate transcription alone or in the presence of tamoxifen. Interestingly, although a high dose of VPA alone inhibited E2-induced transcriptional activity, it increased the ability of tamoxifen to stimulate transcription in the presence of E2. Thus, it is unlikely that VPA enhances the anti-proliferative effect of tamoxifen by increasing the transcription of target genes containing a consensus ERE, where VPA if anything enhances the E2-like effects of tamoxifen.

Since many genes induced by E2 do not have a consensus ERE in their promoters we also examined changes in protein expression from several endogenous E2-regulated genes and receptor levels in MCF-7 cells treated for 72 h with ligands. A therapeutic dose of VPA slightly down-regulated ERα protein expression and also attenuated the increased ERα expression that is typically observed with tamoxifen treatment (Fig. 5b). This effect was observed in the absence of E2 and VPA did not appreciably alter tamoxifen-induced up-regulation of ERα in the presence of E2. ERβ protein was not detected by immunoblotting and its expression was not increased with ligand treatment (data not shown). As expected, cyclin D1 was up-regulated by E2 and down-regulated by tamoxifen, however VPA alone did not alter cyclin D1 expression nor did it alter tamoxifen-mediated down-regulation of cyclin D1. Thus, the effect of VPA on cyclin D1 expression is consistent with the lack of effect on cell cycle progression (Fig. 4a). The pro-survival protein Bcl-2 was up-regulated with E2 treatment and tamoxifen inhibited Bcl-2 stimulation by E2, as expected (Fig. 5b). VPA treatment however did not have a significant effect on Bcl-2 expression either alone or with tamoxifen.

The pro-apoptotic gene Bik has been reported to be an essential mediator of antiestrogen-induced apoptosis in MCF-7 cells, where its expression is up-regulated by E2 withdrawal or by the pure antiestrogen fulvestrant [36]. Under E2-deprived conditions, where Bik

protein is expressed at high levels, VPA and tamoxifen had minimal effects on Bik expression alone and down-regulated Bik when treated together (Fig. 5b). In the presence of E2, where Bik protein is down-regulated, both VPA and antiestrogens individually induced Bik, and co-treatment with both drugs additively enhanced Bik expression at 3 days (Fig. 5b) but antagonized each other's effect on Bik at 6 days (Fig. 5c). It should be noted however that while VPA and antiestrogen treatment did not always synergize to up-regulate Bik, their combined effect on Bik was always increased relative to vehicle alone. In Ishikawa endometrial adenocarcinoma cells neither tamoxifen, raloxifene, or fulvestrant induced Bik expression (Fig. 5c). However, VPA strongly induced Bik in Ishikawa cells when treated alone and in combination with the antiestrogens, thus mirroring the anti-proliferative effect by VPA in these cells.

VPA enhances the efficacy of aromatase inhibitors

Aromatase inhibitors are particularly effective in postmenopausal women whose breast tumors are stimulated by the local production of E2 via aromatase in the breast tissue microenvironment. Aromatase is the enzyme responsible for the conversion of testosterone into E2 and is often overexpressed in breast tumors [37]. To examine the effect of VPA on aromatase inhibitors we treated MCF-7 cells stably overexpressing aromatase (MCF-7aro) and stimulated proliferation with testosterone (Fig. 6a). After 5 days of treatment, testosterone stimulated proliferation as well as E2, indicating that aromatase is functional in MCF-7aro cells and converting testosterone to E2. The aromatase inhibitor letrozole inhibited testosterone-induced proliferation in a dose responsive manner. VPA increased the efficacy of both doses of letrozole tested, inhibiting proliferation better than either drug alone. VPA enhanced the anti-proliferative effect of letrozole in a similar manner when MCF-7aro cells were stimulated with the adrenal androgen androstenedione (Fig. 6b). Taken together, these results indicate VPA cooperates with the antiproliferative effects of the two major forms of hormonal therapy currently used for treating ERα-positive breast cancer, antiestrogens and aromatase inhibitors.

VPA enhances the antiproliferative effect of tamoxifen in MCF-7 cells overexpressing HER-2/neu

Stably overexpresed HER2/neu in MCF-7 cells permits these cells to grow as tamoxifen-resistant tumors in nude mice [38, 39]. Having established that VPA increases the efficacy of tamoxifen in MCF7 cells, we next determined whether VPA could overcome the increased growth rate and decreased sensitivity to tamoxifen typical of MCF-7/HER2 cells growing in vitro (Fig. 7). As expected, we observed an increased basal level of proliferation in MCF-7/HER2 cells when compared to parental MCF-7 cells and a slight decrease in sensitivity to tamoxifen. Like parental cells, VPA inhibited proliferation of MCF-7 cells overexpressing HER2/neu. In the presence of E2, VPA increased the efficacy of tamoxifen in MCF-7/HER2 cells, with the combination of drugs working better than either one alone. These results indicate that VPA enhances the anti-proliferative effect of antiestrogens in both tamoxifen-sensitive cells and in tamoxifen-resistant breast cancer cells.

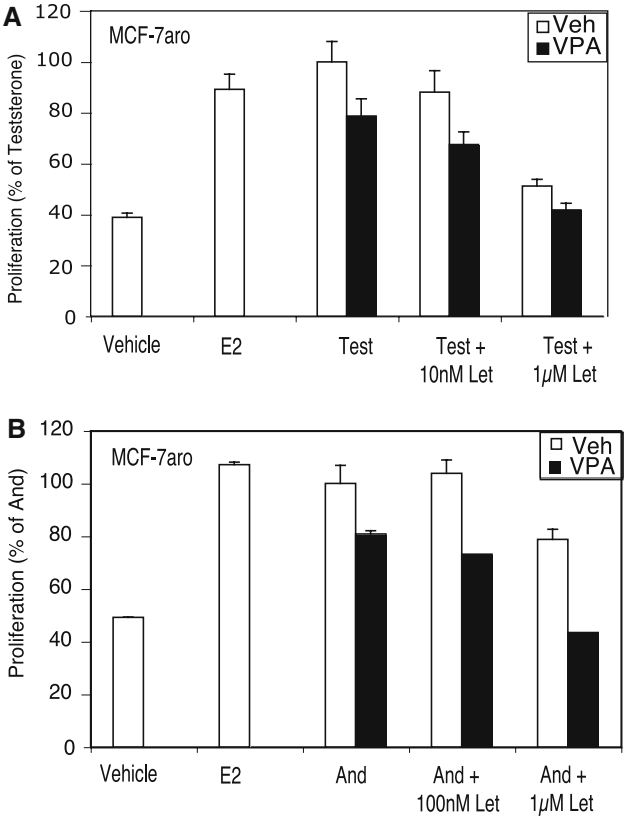


Fig. 6 VPA enhances the efficacy of the aromatase inhibitor letrozole and increases tamoxifen sensitivity in tamoxifen-resistant MCF-7 breast cancer cells overexpressing aromatase (*MCF-7aro*). MCF-7aro cells were stimulated with either 100 nM E2 or 1 nM testosterone (*Test*) (**A**) or 1 μM androstenedione (*And*) (**B**). Stimulated MCF-7aro cells were concurrently treated with the indicated concentrations of letrozole (*Let*) for 5 days with or without 750 μM VPA and proliferation measured using a fluorescent DNA-binding assay. Values represent the percentage of fluorescence relative to testosterone alone and *error bars* represent the SEM from triplicate wells in a representative experiment

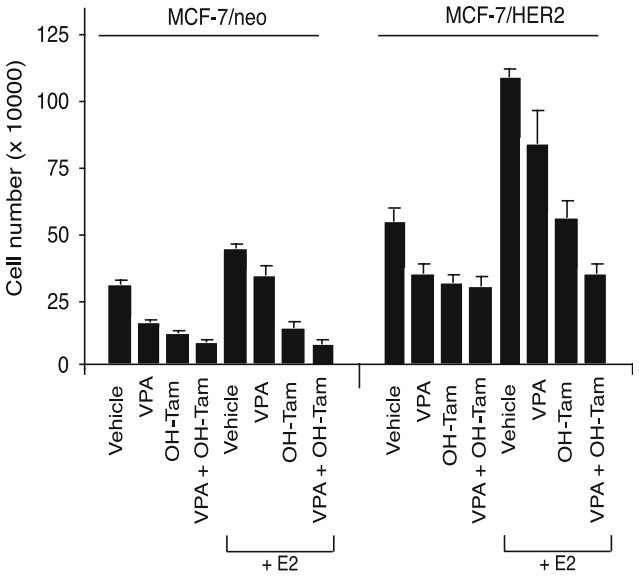


Fig. 7 VPA enhances the efficacy of tamoxifen in MCF-7 cells overexpressing HER2/neu. MCF-7/neo and MCF-7/HER2 cells were treated with 100 pM E2 and either 750 μM VPA and/or 10 nM OH-Tam for 7 days and counted electronically. *Bars* represent the average cell number of three replicates from a representative experiment and *error bars* represent SEM

Discussion

Here we demonstrate that inhibition of HDACs, for example with the inhibitor VPA, cooperates with the two most common hormonal therapies for breast cancer, antiestrogens, and aromatase inhibitors, to achieve an enhanced anti-proliferative effect when treated in combination- one greater than occurs with either agent alone. VPA also sensitizes tamoxifen-resistant MCF-7 cells overexpressing HER2/neu to the anti-proliferative effect of tamoxifen. Interestingly, VPA unexpectedly reverses tamoxifen-induced proliferation in cultured uterine endometrial cells. These cooperative action between VPA, or TSA, or SAHA and antiestrogens to inhibit proliferation in breast cancer cells is likely mediated by their HDAC inhibitory action as these drugs increase acetylated H4 at doses that corresponded to cell inhibition and cooperation with antiestrogens.

The concentration of VPA used in this study that cooperated with anti-hormonal therapy, and also was efficient at inducing histone H4 acetylation, 750 μM, corresponds with target total VPA concentrations in the serum of patients being treated for epilepsy and bipolar disorders (300–850 μM). Whereas the concentration of free VPA, that is, VPA not bound to serum proteins, is likely to be less than half that level, it is noteworthy that a recent phase I/II trial of the combination of VPA and epirubicin for solid tumors observed histone acetylation in blood mononuclear cells and free VPA levels of the order used in this study treating with standard doses of VPA [31].

Our results indicate that VPA enhances the ability of tamoxifen to induce apoptosis but not to arrest cell cycle progression. The pattern of VPA-induced changes in gene expression is consistent with these effects on cell survival since VPA does not attenuate cyclin D1 levels when compared to vehicle or tamoxifen. Both VPA and antiestrogens induce the pro-apoptotic gene Bik. However, when the two drugs are combined they can either cooperate or antagonize each others action depending on the timing of the application. Thus simple synergy of Bik induction cannot fully explain the ability of the two agents to cooperate in the induction of apoptosis. Although we do not currently entirely understand the complexities of regulation of apoptosis in breast cancer cells by antiestrogens and VPA, further investigations into the transcriptional regulation of Bik and other members of the apoptotic pathway are warranted to determine their role in mediating the anti-proliferative effects of anti-cancer drugs in breast cells.

It was recently reported that the HDAC inhibitor TSA sensitized ERα-negative breast cancer cells to the inhibitory effects of tamoxifen and this effect was attributed to the de novo expression of ERβ [12]. The MCF-7 cells used in this study however express very low levels of ERβ protein and treatment with HDAC inhibitors and/or tamoxifen did not increase ERβ protein expression to a detectable level by immunoblotting (data not shown). Thus, it is unlikely that ERβ is a major mediator of the cooperative effects of VPA and tamoxifen in inhibiting ERα-positive cell growth.

Valproic acid and other HDAC inhibitors are known to down-regulate ERα expression and here we find that therapeutic doses of VPA does indeed slightly reduce ERα levels in the absence of E2 [14, 24, 25]. Counterbalancing the decrement in receptor expression, VPA and other HDAC inhibitors increase the action of p160 co-activators for ERα, which have histone acetyltransferase activity, and are in dynamic competition with HDAC-containing complexes. The outcome of these two opposing actions of VPA on modulating ERα activity tends to be variable and is likely gene and tissue-specific. Like other investigators, we also observe that in the absence of E2, higher doses of VPA and TSA increase transcription of an estrogen-responsive ERE reporter gene and enhance the transcriptional activity of tamoxifen [12–14]. However, this increase in transcriptional activity induced by HDAC inhibitors only occurred at very high and cytotoxic doses. In contrast, 750 μM VPA, the therapeutic dose

that effectively inhibited E2-induced proliferation and enhanced the anti-proliferative effects of tamoxifen, had little effect on ERE reporter gene activity. Therefore, alterations in ER-mediated transcription may not be physiologically relevant to the action of low doses of VPA on cell survival, and particular targets, such as Bik expression, may be more predictive for the anti-proliferative effects of HDAC inhibitors.

Valproic acid also enhances tamoxifen sensitivity of MCF-7 cells overexpressing HER2/neu in vitro. Growth factors such as HER2/neu are believed to phosphorylate the ERα and p160 co-activators, resulting in ligand-independent stimulation of target gene expression in these cells [40–42]. Overexpression of HER2/neu has also been causally associated with tamoxifen resistance in the clinic, hypothesized to be due to increased cross-talk between growth factor and ERα pathways [43–45]. The ability of VPA to enhance tamoxifen action on these tamoxifen-resistant cells suggests that VPA might be able to slow the development of tamoxifen resistance, a question we are currently exploring.

The cooperative anti-proliferative effect observed between HDAC inhibitors and anti-hormonal therapies is most likely an additive rather than a synergistic interaction, however it should be noted that in vitro synergy does not necessarily predict therapeutic synergy, defined as a combination of treatments that is more efficacious than using either agent alone [46]. There are several compelling reasons to further investigate combination therapy with VPA and antiestrogens in preclinical tumorigenic animal models and also in the clinic, particularly since VPA and tamoxifen are both well-tolerated clinically approved drugs with known pharmocodynamics and toxicities. VPA has been safely used in the treatment of epilepsy for over 30 years, although some adverse side effects have been reported including teratogenic effects and sometimes intolerable mental actions such as tremor and confusion.

Valproic acid has also been reported to have endocrine-related effects including altered ovarian function and menstrual abnormalities [47–49]. Although we did not observe any stimulatory activity of VPA under our experimental conditions, there have been several reports of selective agonist activity by HDAC inhibitors that warrant attention. Olsen and colleagues report that the sub-therapeutic dose of 100 μM VPA stimulated MCF-7 cell proliferation in the absence of E2, even though it inhibited E2-induced proliferation, an effect we did not observe using our cells and experimental conditions [29]. While our results and that of another group indicate that VPA strongly inhibits endometrial cell growth in vitro, one group reported that VPA stimulates proliferation in Ishikawa cells [50, 51]. Similar doses and treatment regimens were used in each study, however Ishikawa cells are known to be heterogenous and change morphology and receptor status during culture, so it is possible that different cell populations were assayed from ours [52]. In addition, TSA and sodium butyrate have been reported to increase uterine mass and the mitotic index in ovariectomized mice, so it is possible that other HDAC inhibitors such as VPA may have a similar effect [53]. Because of these concerns, in vivo experiments in a preclinical tumorigenic animal model are currently planned to monitor any selective agonist effects in hormone-responsive tissues, in addition to confirming the cooperative anti-proliferative effects between VPA and tamoxifen to inhibit breast tumors.

In summary, combination treatment of the HDAC inhibitor VPA with tamoxifen and other hormonal therapy agents cooperatively inhibits cell proliferation and induces apoptosis in ERα-positive breast cancer cells and in tamoxifen-resistant cells overexpressing HER2/neu. The combination of HDAC inhibitor and antiestrogen therapy could be useful for designing new therapeutic strategies in the future. Particularly attractive is the potential use of HDACs, especially VPA, to increase response to hormonal therapy in ER-positive breast cancer and to increase sensitivity in tamoxifen resistant tumors. Adding VPA to tamoxifen therapy could also protect women from developing uterine adenocarcinoma while enhancing the efficacy of tamoxifen in inhibiting breast tumors.

Acknowledgments We would particularly like to thank Dr. Fred Schaufele for the careful reading of this manuscript and thoughtful suggestions. We also thank Marianna Zavodoskaya, Paul Webb, John Baxter, Michael Campbell, Ira Goldfine, and Jack Youngren for helpful discussions. Financial support was provided by the National Cancer Institute, NIH R01 CA 80210 (PJK) and the California Breast Cancer Research Project, 10FB-0046 (LHG). Peter Kushner has significant financial interests in, and is a former Director and Consultant to, Karobio AB a biotech company that develops ligands for nuclear receptors.

References

1. Clarke R, Skaar TC, Bouker KB et al (2001) Molecular and pharmacological aspects of antiestrogen resistance. J Steroid Biochem Mol Biol 76(1–5):71–84
2. Osborne CK (1998) Tamoxifen in the treatment of breast cancer. N Engl J Med 339(22):1609–1618
3. Ring A, Dowsett M (2004) Mechanisms of tamoxifen resistance. Endocr Relat Cancer 11(4):643–658
4. Fisher B, Costantino JP, Redmond CK et al (1994) Endometrial cancer in tamoxifen-treated breast cancer patients: findings from the National Surgical Adjuvant Breast and

Bowel Project (NSABP) B-14. J Natl Cancer Inst 86(7):527–537
5. Gelber RD, Cole BF, Goldhirsch A et al (1996) Adjuvant chemotherapy plus tamoxifen compared with tamoxifen alone for postmenopausal breast cancer: meta-analysis of quality-adjusted survival. Lancet 347(9008):1066–1071
6. Rivkin SE, Green S, Metch B et al (1994) Adjuvant CMFVP versus tamoxifen versus concurrent CMFVP and tamoxifen for postmenopausal, node-positive, and estrogen receptor-positive breast cancer patients: a Southwest Oncology Group study. J Clin Oncol 12(10):2078–2085
7. Woods KE, Randolph JK Gewirtz DA (1994) Antagonism between tamoxifen and doxorubicin in the MCF-7 human breast tumor cell line. Biochem Pharmacol 47(8):1449–1452
8. De Soto JA, Bowen D, Davis JH et al (2002) Sequence- and time-dependent antagonism between raloxifene and methotrexate in human breast cancer cells. Anticancer Res 22(2A):1007–1009
9. Osborne CK, Kitten L, Arteaga CL (1989) Antagonism of chemotherapy-induced cytotoxicity for human breast cancer cells by antiestrogens. J Clin Oncol 7(6):710–717
10. Johnston SR (2006) Clinical efforts to combine endocrine agents with targeted therapies against epidermal growth factor receptor/human epidermal growth factor receptor 2 and mammalian target of rapamycin in breast cancer. Clin Cancer Res 12(3 Pt 2):1061s–1068s
11. Wyeth Pharmaceuticals (2006) Termination of phase 3 clinical program with oral temsirolimus in women with metastatic breast cancer. From: Medical News Today http://www.medicalnewstoday.com/medicalnews.php?newsid=39843. Cited 21 Mar 2006
12. Jang ER, Lim SJ, Lee ES et al (2004) The histone deacetylase inhibitor trichostatin A sensitizes estrogen receptor alpha-negative breast cancer cells to tamoxifen. Oncogene 23(9):1724–1736
13. Jansen MS, Nagel SC, Miranda PJ et al (2004) Short-chain fatty acids enhance nuclear receptor activity through mitogen-activated protein kinase activation and histone deacetylase inhibition. Proc Natl Acad Sci USA 101(18):7199–7204
14. Margueron R, Duong V, Bonnet S et al (2004) Histone deacetylase inhibition and estrogen receptor alpha levels modulate the transcriptional activity of partial antiestrogens. J Mol Endocrinol 32(2):583–594
15. Villar-Garea A, Esteller M (2004) Histone deacetylase inhibitors: understanding a new wave of anticancer agents. Int J Cancer 112(2):171–178
16. Marks P, Rifkind RA, Richon VM et al (2001) Histone deacetylases and cancer: causes and therapies. Nat Rev Cancer 1(3):194–202
17. Glaser KB, Staver MJ, Waring JF et al (2003) Gene expression profiling of multiple histone deacetylase (HDAC) inhibitors: defining a common gene set produced by HDAC inhibition in T24 and MDA carcinoma cell lines. Mol Cancer Ther 2(2):151–163
18. Richon VM, Sandhoff TW, Rifkind RA et al (2000) Histone deacetylase inhibitor selectively induces p21WAF1 expression and gene-associated histone acetylation. Proc Natl Acad Sci USA 97(18):10014–10019
19. Munster PN, Troso-Sandoval T, Rosen N et al (2001) The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces differentiation of human breast cancer cells. Cancer Res 61(23):8492–8497
20. Kim MS, Blake M, Baek JH et al (2003) Inhibition of histone deacetylase increases cytotoxicity to anticancer drugs targeting DNA. Cancer Res 63(21):7291–7300
21. Castro-Galache MD, Ferragut JA, Barbera VM et al (2003) Susceptibility of multidrug resistance tumor cells to apoptosis induction by histone deacetylase inhibitors. Int J Cancer 104(5):579–586
22. Vigushin DM, Ali S, Pace PE et al (2001) Trichostatin A is a histone deacetylase inhibitor with potent antitumor activity against breast cancer in vivo. Clin Cancer Res 7(4):971–976
23. Margueron R, Licznar A, Lazennec G et al (2003) Oestrogen receptor alpha increases p21(WAF1/CIP1) gene expression and the antiproliferative activity of histone deacetylase inhibitors in human breast cancer cells. J Endocrinol 179(1):41–53
24. Reid G, Metivier R, Lin CY et al (2005) Multiple mechanisms induce transcriptional silencing of a subset of genes, including oestrogen receptor alpha, in response to deacetylase inhibition by valproic acid and trichostatin A. Oncogene 24(31):4894–4907
25. Alao JP, Lam EW, Ali S et al (2004) Histone deacetylase inhibitor trichostatin A represses estrogen receptor alpha-dependent transcription and promotes proteasomal degradation of cyclin D1 in human breast carcinoma cell lines. Clin Cancer Res 10(23):8094–8104
26. Phiel CJ, Zhang F, Huang EY et al (2001) Histone deacetylase is a direct target of valproic acid, a potent anticonvulsant, mood stabilizer, and teratogen. J Biol Chem 276(39):36734–36741
27. Gottlicher M, Minucci S, Zhu P et al (2001) Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. Embo J 20(24):6969–6978
28. Blaheta RA, Michaelis M, Driever PH et al (2005) Evolving anticancer drug valproic acid: insights into the mechanism and clinical studies. Med Res Rev 25(4):383–397
29. Olsen CM, Meussen-Elholm ET, Roste LS et al (2004) Antiepileptic drugs inhibit cell growth in the human breast cancer cell line MCF7. Mol Cell Endocrinol 213(2):173–179
30. Marchion DC, Bicaku E, Daud AI et al (2005) In vivo synergy between topoisomerase II and histone deacetylase inhibitors: predictive correlates. Mol Cancer Ther 4(12):1993–2000
31. Munster PN, Minton SE, Marchion DC, Carter W, Bicaku E, Padilla B, Lush R, Sullivan DM, Daud AI (2006) Phase I/II trial and pharmacokinetic/pharmacodynamic analysis exploring a synergistic interaction between the histone deacetylase inhibitor, valpoic acid and the anthracycline, epirubicin. Presented at the San Antonio Breast Cancer Symposium, San Antonio, TX
32. Liu MM, Albanese C, Anderson CM et al (2002) Opposing action of estrogen receptors alpha and beta on cyclin D1 gene expression. J Biol Chem 277(27):24353–24360
33. Webb P, Nguyen P, Valentine C et al (1999) The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. Mol Endocrinol 13(10):1672–1685
34. Vermes I, Haanen C, Steffens-Nakken H et al (1995) A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 184(1):39–51
35. Webb P, Nguyen P, Kushner PJ (2003) Differential SERM effects on corepressor binding dictate ERalpha activity in vivo. J Biol Chem 278(9):6912–6920
36. Hur J, Chesnes J, Coser KR et al (2004) The Bik BH3-only protein is induced in estrogen-starved and antiestrogen-exposed breast cancer cells and provokes apoptosis. Proc Natl Acad Sci USA 101(8):2351–2356

37. Larionov AA, Berstein LM, Miller WR (2002) Local uptake and synthesis of oestrone in normal and malignant postmenopausal breast tissues. J Steroid Biochem Mol Biol 81(1):57–64
38. Benz CC, Scott GK, Sarup JC et al (1993) Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. Breast Cancer Res Treat 24(2):85–95
39. Kurokawa H, Lenferink AE, Simpson JF et al (2000) Inhibition of HER2/neu (erbB-2) and mitogen-activated protein kinases enhances tamoxifen action against HER2-overexpressing, tamoxifen-resistant breast cancer cells. Cancer Res 60(20):5887–5894
40. Kato S (2001) Estrogen receptor-mediated cross-talk with growth factor signaling pathways. Breast Cancer 8(1):3–9
41. Lopez GN, Turck CW, Schaufele F et al (2001) Growth factors signal to steroid receptors through mitogen-activated protein kinase regulation of p160 coactivator activity. J Biol Chem 276(25):22177–22182
42. Bunone G, Briand PA, Miksicek RJ et al (1996) Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. Embo J 15(9):2174–2183
43. Nicholson RI, Hutcheson IR, Harper ME et al (2002) Modulation of epidermal growth factor receptor in endocrine-resistant, estrogen-receptor-positive breast cancer. Ann N Y Acad Sci 963:104–115
44. Osborne CK, Schiff R (2003) Growth factor receptor cross-talk with estrogen receptor as a mechanism for tamoxifen resistance in breast cancer. Breast 12(6):362–367
45. Shou J, Massarweh S, Osborne CK et al (2004) Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst 96(12):926–935
46. Greco WR, Faessel H, Levasseur L (1996) The search for cytotoxic synergy between anticancer agents: a case of Dorothy and the ruby slippers? J Natl Cancer Inst 88(11):699–700
47. Isojarvi JI, Tauboll E, Herzog AG (2005) Effect of antiepileptic drugs on reproductive endocrine function in individuals with epilepsy. CNS Drugs 19(3):207–223
48. O'Donovan C, Kusumakar V, Graves GR et al (2002) Menstrual abnormalities and polycystic ovary syndrome in women taking valproate for bipolar mood disorder. J Clin Psychiatry 63(4):322–330
49. Perucca E (2005) Birth defects after prenatal exposure to antiepileptic drugs. Lancet Neurol 4(11):781–786
50. Takai N, Desmond JC, Kumagai T et al (2004) Histone deacetylase inhibitors have a profound antigrowth activity in endometrial cancer cells. Clin Cancer Res 10(3):1141–1149
51. Graziani G, Tentori L, Portarena I et al (2003) Valproic acid increases the stimulatory effect of estrogens on proliferation of human endometrial adenocarcinoma cells. Endocrinology 144(7):2822–2828
52. Nishida M, Kasahara K, Oki A et al (1996) Establishment of eighteen clones of Ishikawa cells. Hum Cell 9(2):109–116
53. Gunin AG, Kapitova IN, Suslonova NV (2005) Effects of histone deacetylase inhibitors on estradiol-induced proliferation and hyperplasia formation in the mouse uterus. J Endocrinol 185(3):539–549